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The β1-integrin-p-FAK-p130Cas-DOCK180-RhoA-vinculin is a novel regulatory protein complex at the apical ectoplasmic specialization in adult rat testes

Michelle K.Y. Siu,[1,†,*] Ching Hang Wong,[1] Weiliang Xia,[1,‡] Dolores D. Mruk,[1] Will M. Lee[2] and C. Yan Cheng[1,*]

[1]Center for Biomedical Research; The Population Council; New York, NY USA; [2]School of Biological Sciences; University of Hong Kong; Hong Kong, China

Current addresses: [†]Department of Pathology; Faculty of Medicine; University of Hong Kong; Hong Kong, China; [‡]Med-X Research Institute, Shanghai Jiao Tong University, Shanghai, China

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During spermatogenesis, step 1 spermatids (round spermatids) derive from spermatocytes following meiosis I and II at stage XIV of the epithelial cycle begin a series of morphological transformation and differentiation via 19 steps in rats to form spermatozoa. This process is known as spermiogenesis, which is marked by condensation of the genetic material in the spermatid head, formation of the acrosome and elongation of the tail. Since developing spermatids are lacking the robust protein synthesis and transcriptional activity, the cellular, molecular and morphological changes associated with spermiogenesis rely on the Sertoli cell in the seminiferous epithelium via desmosome and gap junction between Sertoli cells and step 1–7 spermatids. Interestingly, a unique anchoring junction type arises at the interface of step 8 spermatid and Sertoli cell known as apical ectoplasmic specialization (apical ES). Once it appears, apical ES is the *only* anchoring device restricted to the interface of step 8–19 spermatids and Sertoli cells to confer spermatid polarity, adhesion, signal communication and structural support, and to provide nutritional support during spermiogenesis, replacing desmosome and gap junction. While the adhesion protein complexes that constitute the apical ES are known, the signaling protein complexes that regulate apical ES dynamics, however, remain largely unknown. Herein we report the presence of a FAK (focal adhesion kinase)-p130Cas (p130 Crk-associated substrate)-DOCK180 (Dedicator of cytokinesis 180)-RhoA (Ras homolog gene family, member A)-vinculin signaling protein complex at the apical ES, which is also an integrated component of the β1-integrin-based adhesion protein complex based on co-immunoprecipitation experiment. It was also shown that besides p-FAK-Tyr^{397} and p-FAK-Tyr^{576}, β1-integrin, p130Cas, RhoA and vinculin displayed stage-specific expression in the seminiferous epithelium during the epithelial cycle with predominant localization at the apical ES as demonstrated by immunohistochemistry. Based on these findings, functional studies can now be performed to assess the role of this β1-integrin-p-FAK-p130Cas-DOCK180-RhoA-vinculin protein complex in apical ES dynamics during spermiogenesis.

Introduction

During spermatogenesis in adult rat testes, late primary spermatocytes undergo meiosis I, to be followed by meiosis II, in the apical (adluminal) compartment of the seminiferous epithelium at stage XIV of the epithelial cycle behind the blood-testis barrier (BTB), giving rise to haploid round spermatids (step 1 spermatids).[1-3] Once formed, round spermatids undergo considerable morphological changes, differentiating from step 1 through 19 spermatids in rats (16 and 6 steps in mice and men, respectively), via spermiogenesis.[1,4] Spermiogenesis is typified by the condensation of nuclear materials in the spermatid head, the development of acrosome above the head region, the packaging of the mitochondria in the mid-piece, the elongation of the tail and the shedding of the unwanted cytosolic materials as the residual body to be engulfed by Sertoli cells, so that fully developed spermatids (spermatozoa) can be released from the seminiferous epithelium at spermiation.[5-7] However, developing spermatids remain attached to the Sertoli cell in the seminiferous epithelium until spermiation to obtain the needed nourishments, structural and hormonal supports from Sertoli cells. From steps 8 through 19 elongating spermatids, these differentiating cells remain anchored onto the Sertoli cell via a testis-specific atypical adherens junction (AJ) type known as apical ectoplasmic specialization (apical ES).[8-11] Once the apical ES appears, this is the only anchoring device at the Sertoli cell-elongating spermatid interface until it undergoes degeneration shortly before spermiation that take place at stage VIII of the epithelial cycle.[4,12,13] Besides conferring cell adhesion

*Correspondence to: Michelle K.Y. Siu and C. Yan Cheng; Email: mkysiu@pathology.hku.hk and Y-Cheng@popcbr.rockefeller.edu
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Table 1. Antibobies that were used for different experiments in this report

Antibody	Vendor	Catalog no.	Uses*
Rabbit anti-β1-integrin	Millipore (formerly Chemicon)	AB1952	IP, IB (1:1,000), IHC (1:100)
Rabbit anti-FAK	Santa Cruz Biotechnology (Santa Cruz, CA)	sc-558	IP, IB (1:400)
Rabbit anti-p-FAK-Tyr^{397}	Upstate Biotechnology (Lake Placid, NY)	07-012	IP, IB (1:1,000)
Rabbit anti-p-FAK-Tyr^{576}	Upstate Biotechnology	07-157	IB (1:1,000)
Rabbit anti-p130 Cas	Santa Cruz Biotechnology	sc-859	IP, IB (1:200), IHC (1:100)
Rabbit anti-p-p130 Cas Tyr^{410}	Cell Signaling Technology (Beverly, MA)	4011	IB (1:1,000)
Mouse anti-Crk	BD Transduction Laboratories (San Jose, CA)	610036	IP, IB (1:1,000)
Goat anti-DOCK180	Santa Cruz Biotechnology	sc-6167	IB (1:200)
Goat anti-R-ras	Santa Cruz Biotechnology	sc-32029	IB (1:200)
Rabbit anti-Rho A	Upstate Biotechnology	05-778	IP, IB (1:1,000), IHC (1:100)
Rabbit anti-Grb2	Santa Cruz Biotechnology	sc-255	IB (1:200)
Mouse anti-vinculin	Aldrich-Sigma (St. Louis, MO)	V9193	IP, IB (1:1,000), IHC (1:200)
Goat anti-nectin-3	Santa Cruz Biotechnology	sc-14806	IP (1:400)
Goat anti-actin	Santa Cruz Biotechnology	sc-1616	IB (1:200)

*IB, immunoblotting; IP, immunoprecipitation; IHC, immunohistochemistry; IF, immunofluorescence microscopy. Antibody dilutions are indicated in bracket. These antibodies cross-reacted with the corresponding proteins in rats as indicated by the manufacturers/vendors.

function, apical ES also maintains proper orientation of developing spermatids so that the heads of elongating spermatids are all pointing towards the basement membrane.[8,14] Since this is the only anchoring device remaining at the Sertoli cell-spermatid interface from step 8–19 spermatids, it is obvious that the apical ES also serves as a platform for signal transduction and/or communication between Sertoli cells and spermatids to coordinate and synchronize various cellular events during spermiogenesis. This thus illustrates the significance of apical ES during spermiogenesis in the seminiferous epithelium.

Based on studies in the past decade, several structural proteins have been identified to be the putative constituents of the apical ES, including the integrin/laminin, the nectin/afadin, and the cadherin/catenin protein complexes; with the integrin/laminin complex being one of the best studied cell adhesion complexes at the apical ES.[4,10,15] However, peripheral proteins in particular nonreceptor protein kinases and adaptors that regulate the adhesion properties of these protein complexes remain largely unknown. This thus severely hampered our efforts to design functional experiments to investigate the mechanism(s) by which these cell adhesion proteins, such as the integrin/laminin protein complex, regulate apical ES adhesion and polarity function during spermiogenesis. In recent years, some of the proteins that are known to be confined to cell-matrix interface at the focal contact (also known as focal adhesion complex, an actin-based cell-matrix anchoring junction type) to facilitate cell movement were found to be components of the apical ES, such as FAK, ILK (integrin-linked kinase) and vinculin.[16-18] Additionally, gap junction proteins (e.g., connexin 43,[19]) and tight junction proteins (e.g., JAM-C, junctional adhesion molecule-C also known as JAM-1),[20] coxsackie and adenovirus receptor (CAR),[21,22] are also found to be integrated components of the apical ES, making it a hybrid anchoring junction type.[4,8,15,23] However, a detailed analysis on the components of the integrin-based cell adhesion protein complex which regulate signaling function at the apical ES during spermatogenesis, such as spermiogenesis and spermiation is still lacking. Earlier studies from our laboratory have shown that β1-integrin at the apical ES forms a bona fide complex with FAK,[16] in particular p-FAK-Tyr^{397} and p-FAK-Tyr^{576}, we thus thought it pertinent to perform a careful analysis to identify the peripheral proteins that are the integrated components of this β1-integrin-FAK-based cell adhesion protein complex at the apical ES. Once this information is known, it will facilitate the design of functional studies to delineate the regulation of apical ES dynamics during spermiogenesis. This is the subject of the present report.

Results

Analysis of the binding partners of β1-integrin and the p130Cas/Crk/DOCK180 protein complex at the apical ES in adult rat testes. To gain a better insight on the components of the β1-integrin-based cell adhesion protein complex at the apical ES, Co-IP (co-immunoprecipitation) was performed using lysates of seminiferous tubules isolated from 90-day-old rats (~250-270 gm body weight, b.w.) with negligible contaminations of Leydig and peritubular myoid cells using antibodies against β1-integrin, p-FAK-Tyr^{397}, p130Cas, vinculin, RhoA and nectin-3 (**Table 1**). It is noted that β1-integrin is localized predominantly at the apical ES and is a putative apical ES component.[24-26] As shown in **Figure 1A**, β1-integrin was found to structurally associate with p-FAK-Tyr^{397}, p130Cas, vinculin and RhoA but not nectin-3. We also investigated the presence of the p130Cas/Crk/DOCK180 complex at the apical ES. Antibodies against β1-integrin, p130Cas, Crk and DOCK180 were used. It was shown that the p130Cas/Crk/DOCK180 complex was found at the apical ES in the testis and was structurally associated with β1-integrin (**Fig. 1B**). No detectable band was detected in negative controls using normal rabbit or mouse IgG for immunoprecipitation (data not shown). Positive controls shown in **Figure 1A and B** were

lysates of testes used for immunoblottings without subjected to immunoprecipitation.

Relative protein levels of the components of the β1-integrin-pFAK-Tyr^{397}-p130Cas-RhoA-vinculin protein complex in sertoli and germ cells. To determine the endogenous levels of the components of the integrin-based cell adhesion protein complex, including β1-integrin, FAK, p-FAK-Tyr^{397}, p-FAK-Tyr^{576}, p130Cas, Crk, DOCK180, R-ras, RhoA, Grb2 and vinculin, in Sertoli and germ cells, protein lysates (about 50 μg total protein per lane) were used from 20-day-old Sertoli cells cultured for 5 days alone (20D SC, **Fig. 2A**) and also from total germ cells isolated from 90-day-old rats (90D GC, **Fig. 2A**) cultured for <12-hr, for immunoblot analysis. All the target proteins were detected in Sertoli and germ cells, except for Crk (an adaptor protein, also an oncogene, encoding an activator of tyrosine protein kinase originally identified in chicken sarcoma), DOCK180 and Grb2 (growth factor receptor-bound protein 2), which apparently was only restricted to Sertoli cells. Higher protein levels of β1-integrin, p-FAK-Tyr^{397}, p-FAK-Tyr^{576} and RhoA (**Fig. 2A**) were found in 90-day-old germ cells than in 20-day-old Sertoli cells, whereas Crk, DOCK180, R-ras (Ras-related protein R-ras, an atypical member of the Ras subfamily of small GTPases), Grb2 and vinculin (**Fig. 2A**) were more predominant in 20-day-old Sertoli cells. A relatively similar FAK and p130Cas (**Fig. 2A**) protein level was detected in both Sertoli and germ cells. It is noted that Sertoli cells isolated from 20-day-old rats are differentiated, ceased to divide, and indistinguishable from Sertoli cells isolated from adult rat testes functionally and physiologically based on earlier studies.[66,67]

Changes in the endogenous levels of the components of the β1-integrin-pFAK-Tyr^{397}-p130Cas-RhoA-vinculin protein complex and the Rho A activation during anchoring junction assembly in Sertoli-germ cell cocultures. To determine the involvement of the components of the β1-integrin-pFAK-Tyr^{397}-p130Cas-RhoA-vinculin protein complex in Sertoli-germ cell anchoring junction (e.g., apical ES) assembly, protein lysates obtained from cocultures were subjected to immunoblottings for analysis. In short, Sertoli cells were cultured alone for 3 days to from an intact cell epithelium, thereafter, total germ cells isolated from adult rat testes were added onto the Sertoli cell epithelium on day 4 to initiate the assembly of Sertoli-germ cell anchoring junctions, including desmosome, gap junction and apical ES; but since β1-integrin, p-FAK-Tyr^{397}, p-FAK-Tyr^{576}, and their associated proteins are restricted to the apical ES as shown herein and earlier described,1[6,18,24,65] changes reported herein involving proteins pertinent to anchoring junction assembly are limited to the apical ES. The protein levels of β1-integrin, p-FAK-Tyr^{397}, p-FAK-Tyr^{576}, Crk, DOCK180, R-ras, RhoA, Grb2 and vinculin (**Fig. 2A and B**) along with Rho A activity was induced at the time germ cell attach to the Sertoli cell epithelium, preparing for anchoring junctions, most notably apical ES, formation.[27] It is noted that functional apical ES was established in these cocultures by ~24-48 hr when examined by electron microscopy as described.[65,79,80] Most target proteins reduced or returned to their basal level when functional apical ES and desmosomes were formed by 2 d, except for vinculin, which remained high at 2 d (**Fig. 2A and B**). The protein levels of FAK and p130Cas remained relatively stable throughout the entire experiment (**Fig. 2A and B**).

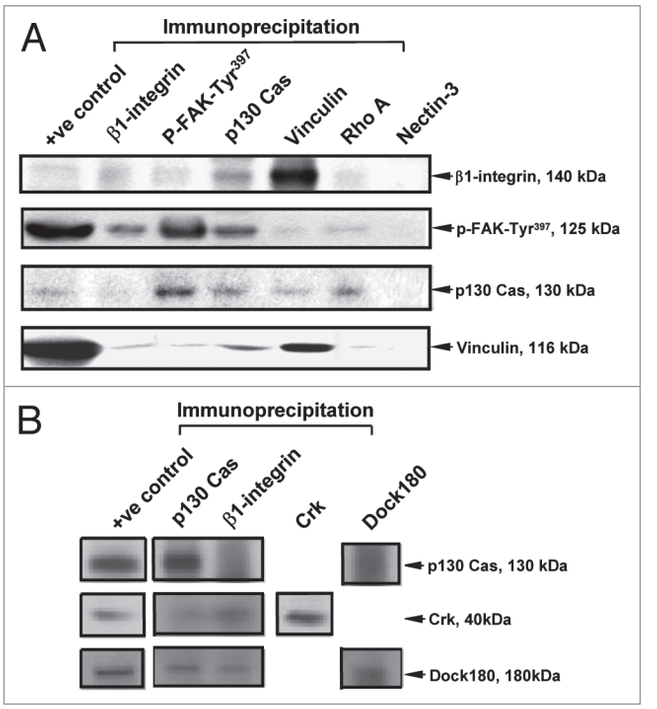

Figure 1. A study to identify the binding partners of β1-integrin-based adhesion protein complex at the apical ES of adult rat testes. Co-IP was performed using lysates of seminiferous tubules (~500 μg proteins per sample) as described in Materials and Methods, and the immunocomplexes were then subjected to immunoblotting (IB) using different specific antibodies against β1-integrin, p-FAK-Tyr^{397}, p130Cas and vinculin in (A) and p130Cas, Crk and Dock180 in (B). This experiment was repeated three times using different batches of samples with similar results. Positive control indicates lysates alone without subjected to immunoprecipitation. Negative controls included the use of normal rabbit or mouse IgG.

Stage-specific expression of β1-integrin, p130Cas, RhoA and vinculin in the seminiferous epithelium of normal rat testes. *Stage-specific localization of β1-integrin.* To localize β1-integrin in normal rat testes at different stages of the epithelial cycle, immunohistochemistry were performed using frozen sections. Immunoreactive β1-integrin appears as reddish-brown precipitate was detected at the apical ES, displaying a stage-specific expression being highest in early stage VIII just prior to spermiation (**Fig. 3A**) with relatively little β1-integrin detected in the seminiferous epithelium at the basal compartment near the basement membrane possibly at the site of hemidesmosomes (an intermediate filament-based cell-matrix anchoring junction type in the testis), but virtually none at the site of the BTB, such as the basal ES (**Fig. 3B**). These findings are consistent with a recent report using dual-labeled immunofluorescence analysis to assess the localization of β1-integrin in the adult rat testis.[81] Immunoreactive β1-integrin was found at the apical ES as early as in stage X (**Fig. 3C**) when apical ES was assembled between Sertoli cells

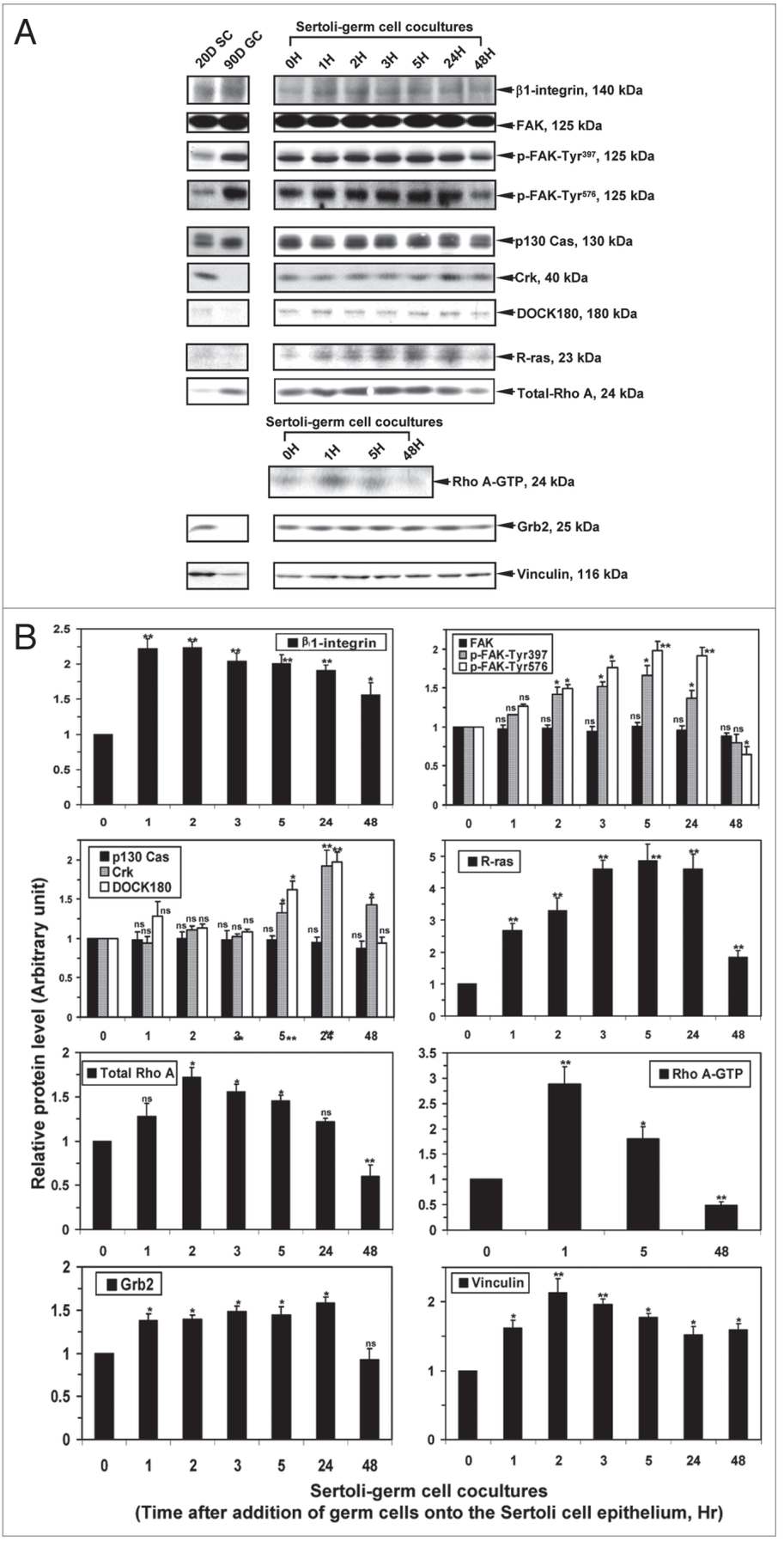

Figure 2. A study to assess changes in the steady-state levels of component proteins of the β1-integrin-pFAK-Tyr^{397}-p130Cas-RhoA-vinculin protein complex and Rho A activity during Sertoli-germ cell anchoring junction assembly. Sertoli cells were cultured alone for 3 days at a density of 0.5 x 10^6 cells/cm^2 on Matrigel-coated dishes forming an intact cell epithelium with the functional TJ-permeability barrier that mimics the BTB in vivo. On day 4, freshly isolated total germ cells at a relative ratio of spermatogonia:spermatocytes:round spermatids:elongating/elongated spermatids similar to those found in vivo were added onto the Sertoli epithelium to initiate anchoring junction assembly which usually completed by 1–2 day when examined by electron microscopy[65,79,80] illustrating the presence of apical ES and desmosome at the Sertoli-germ cell interface. However, as noted in the findings reported herein and shown in **Figures 3-6**, both ß1-integrin and its associated signaling proteins are spatially restricted to the apical ES in the seminiferous epithelium, changes in the steady-state protein levels reported herein reflected changes during the assembly of apical ES in these cocultures. At specified time points, cocultures were terminated for lysate preparation for immunoblot analysis using different antibodies and Rho A activation assay. Immunoblots on the left panel in (A)represent Sertoli and germ cells terminated on day 4 and ~12-hr after their cultures in vitro, respectively, to assess the relative steady-state levels of different apical ES proteins. The bar graph shown in (B) represents composite data from three independent experiments using immunoblots such as those shown in (A). *significantly different by ANOVA, $p < 0.05$; **$p < 0.01$; ns, not significantly different, nd, non-detectable.

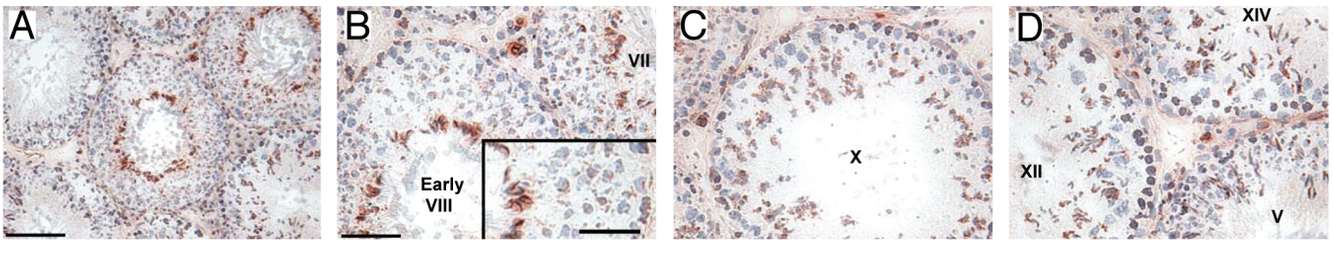

Figure 3. Stage-specific localization of β1-integrin in the seminiferous epithelium of adult rat testes. Frozen cross-sections (~6 μm thick) of adult rat testes were stained for β1-integrin by immunohistochemistry as described in Materials and Methods. Immunoreactive β1-integrin appears as reddish-brown precipitate. (A) is the cross-section of an adult rat testis showing β1-integrin immunoreactive substances in the seminiferous epithelium at low magnification. (B–D) are representative cross-sections of tubules at stages VII, VIII (B), X (C) and XII, XIV, V (D) of the epithelial cycle. The boxed area shown in (B) is a selected region of the seminiferous epithelium at higher magnification. These micrographs, similar to all other immunohistochemistry experiments, are representative results of a single experiment which was repeated three times using different testes from different adult rats, which yielded similar results. Bar in (A) = 120 mm; bar in (B) = 50 mm, which also applies to (C and D); bar in boxed area in (B) = 25 μm.

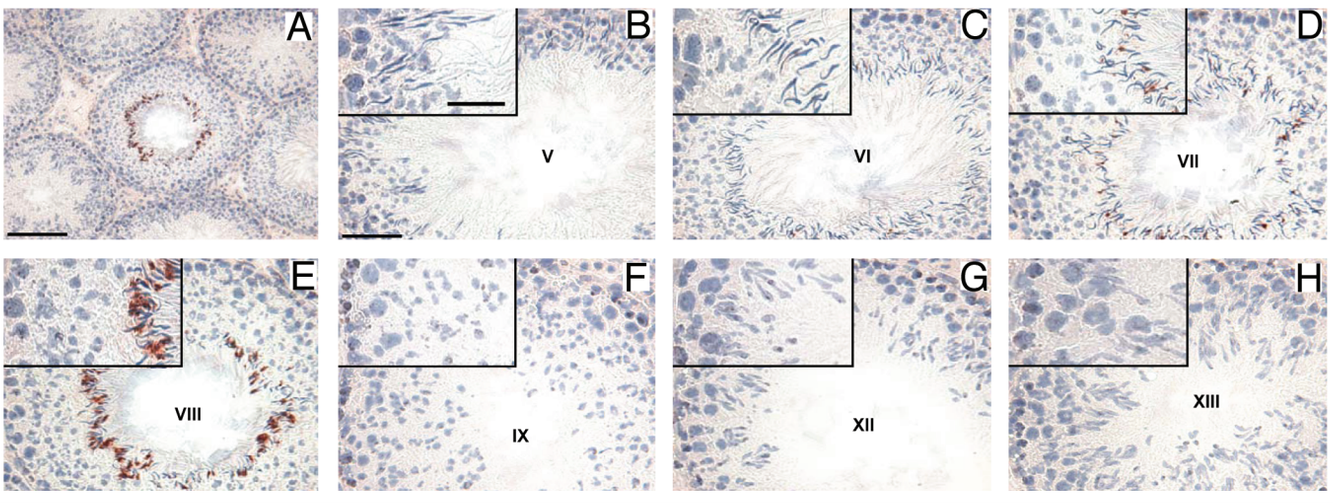

Figure 4. Stage-specific localization of p130Cas in the seminiferous epithelium of adult rat tests. Experimental conditions that were used to localize p130Cas (and for Rho A and vinculin shown in **Figs. 5 and 6**, respectively) in the seminiferous epithelium of rat testes by immunohistochemistry were similar to β1-integrin (see legend to Fig. 3). (A) is the cross-section of an adult rat testis showing p130Cas immunoreactive at low magnification. (B–H) are representative cross-sections of tubules at stages V (B), VI (C), VII (D), VIII (E), IX (F), XII (G) and XIII (H) of the seminiferous epithelial cycle. The boxed areas shown in (B-H) are selected regions of the seminiferous epithelium at higher magnification, illustrating the stage-specific localization of p130Cas in rat testes. Bar in (A) = 120 μm; bar in (B) = 50 μm, which also applies to (C–H); bar in boxed area in (B) = 20 μm, which applies to all boxed areas in (C–H).

and step 8 spermatids, which persisted through stages XI-V (**Fig. 3D**) and strongest at early stage VIII (**Fig. 3C**).

Stage-specific localization of p130Cas. p130Cas appears as reddish-brown precipitate at the apical ES in the seminiferous epithelium of adult rat testes beginning only in stage VII tubules (**Fig. 4A–C** versus **D**) which became considerably intensified at stage VIII (**Fig. 4E**) but diminished to a level that was virtually undetectable in stage IX (**Fig. 4F**) and also not detectable in remaining stages (**Fig. 4G and H**). In short, p130Cas began to express at the apical ES at stage VII tubules and greatly intensified at early stage VIII.

Stage-specific localization of Rho A. Localization of RhoA in the seminiferous epithelium of adult rat testes using frozen sections for immunohistochemistry, similar to β1-integrin and p130Cas shown in **Figures 3 and 4**, was also stage-specific, being restricted mostly to the apical ES (**Fig. 5A–G**) and highest in stage VIII tubules (**Fig. 5A and D** versus **B, C and E–G**).

Stage-specific localization of vinculin. Localization of vinculin in the seminiferous epithelium of adult rat testes using paraffin sections for immunohistochemistry was also shown to be stage-specific, however, unlike β1-integrin, p130Cas and RhoA which are restricted almost exclusively to the apical ES (see **Figs. 3–5**), vinculin was found at the apical ES, but also the basal ES at the BTB near the basement membrane (**Fig. 6A–F**). Immunoreactive vinculin appears as reddish-brown precipitate at the apical ES in stage V tubules, and its intensity at the apical ES increased steadily from stage VI through early VIII, but diminished by late stage VIII at the time of spermiation (**Fig. 6A–F**). Vinculin

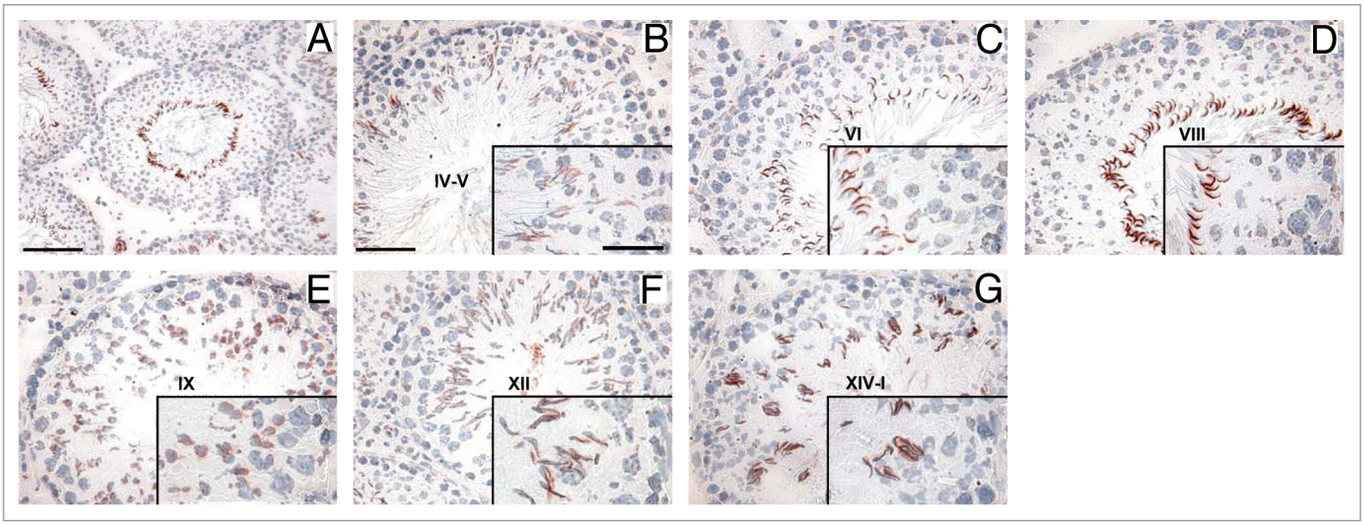

Figure 5. Stage-specific localization of RhoA in the seminiferous epithelium of adult rat testes. (A) is the cross-section of an adult rat testis showing the localization of RhoA immunoreactive substances in the seminiferous epithelium at low magnification using frozen sections. (B–H) are representative cross-sections of tubules at stages IV-V (B), VI (C), VIII (D), IX (E), XII (F) and XIV-I (G) of the seminiferous epithelial cycle. The boxed areas shown in (B–G) are magnified areas of the seminiferous epithelium, illustrating the localization of RhoA at the convex side of the spermatid head, consistent with its presence at the apical ES. Bar in (A) = 120 μm; bar in (B) = 50 μm, which also applies to (C–G); bar in boxed area in (B) = 20 μm, which applies to boxed areas in (C–G).

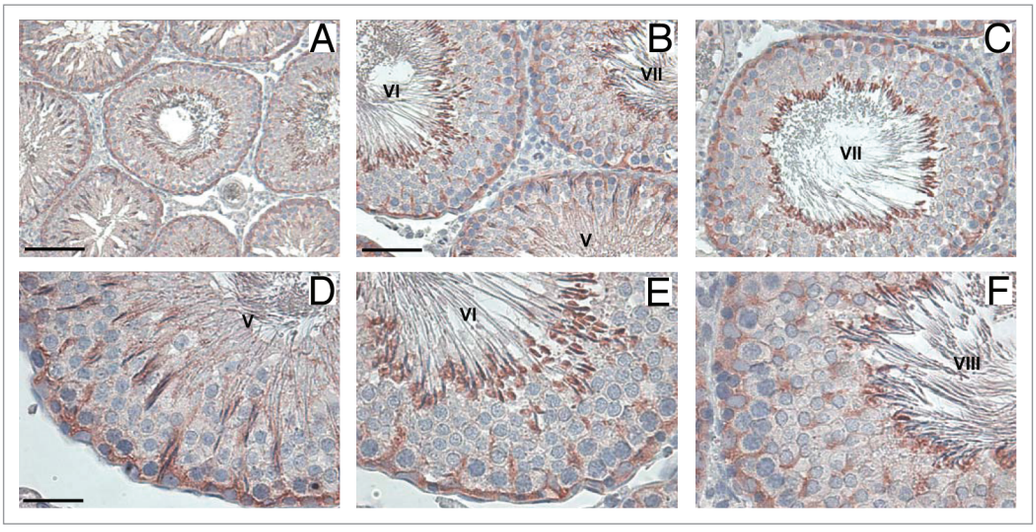

Figure 6. Stage-specific localization of vinculin in the seminiferous epithelium of adult rat testes. (A) is the cross-section of an adult rat testis showing vinculin immunoreactive substances in the seminiferous epithelium at low magnification using paraffin sections. (B–F) are representative cross-sections of tubules at stages V-VII (B), VII (C), V (D), VI (E) and VIII (F) of the epithelial cycle. Bar in (A) = 120 μm, bar in (B) = 50 μm, which also applies to (C); bar in (D) = 20 μm, which also applies to (E and F).

was also detected at the basal region of the epithelium, consistent with its localization at the BTB, however, its intensity diminished considerably at the BTB at stage VIII prior to spermiation (**Fig. 6D and E** versus **F**).

Changes in the endogenous levels of the components of the β1-integrin-pFAK-Tyr^{397}-p130Cas-RhoA-vinculin protein complex and the Rho A activation during adjudin-induced anchoring junction disruption in the testis in vivo. To induce progressive loss of germ cells from the seminiferous epithelium by disrupting anchoring junctions between Sertoli and germ cells in the testis initially and most notably at the apical ES to be followed by the desmosome and gap junction,[28] adult rats were treated with a single dose of adjudin (50 mg/kg b.w.) by gavage. A steady induction in the protein levels of β1-integrin and vinculin from the beginning of the treatment to 96 h, peaked at 96 h, was noted with a mild reduction by 15 d (**Fig. 7A and B**). A transient induction in the protein levels of p-FAK-Tyr^{397}, p-FAK-Tyr^{576}, p130Cas, p-p130Cas-Tyr^{410}, Crk, R-ras, RhoA, Grb2 from the beginning of the treatment to 8 h, peaked at 2–4 h, was detected (**Fig. 7A and B**). The induced p-FAK-Tyr^{397}, p-FAK-Tyr^{576},

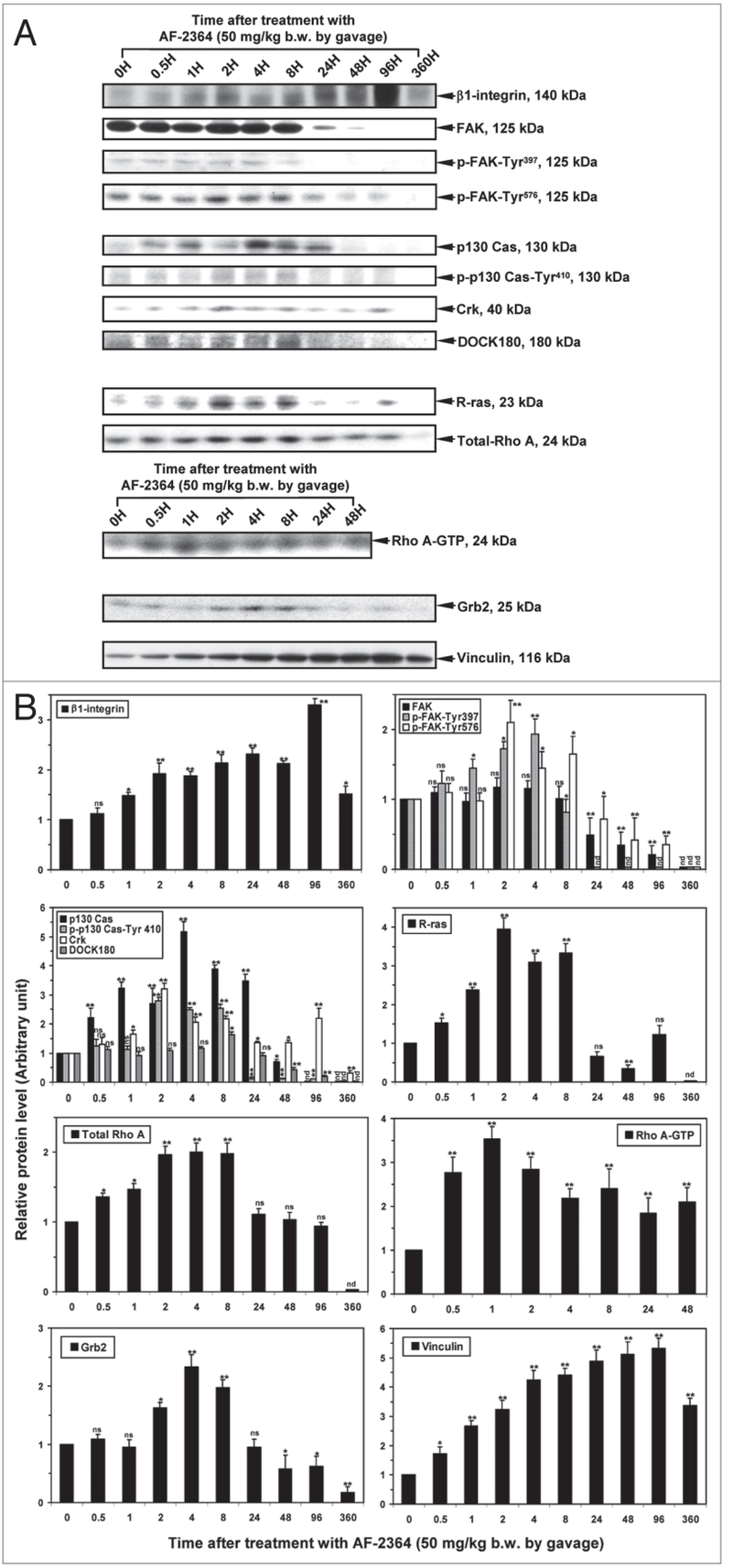

Figure 7. Changes in the steady-state levels of proteins at the apical ES during adjudin-induced anchoring junction restructuring and germ cell loss from the seminiferous epithelium in adult rat testes. (A) Adult rats (~300 gm b.w.) were treated with a single dose of adjudin (50 mg/kg. b.w.) by gavage which is known to induce anchoring junction restructuring in the seminiferous epithelium leading to elongating spermatid depletion from the epithelium as a result of apical ES disruption. At specified time points, rats were euthanized by CO_2 asphyxiation, lysates were prepared from rat testes for immunoblot analysis and Rho A activation assay, illustrating changes in the steady-state protein levels of the components of β1-integrin-based apical ES protein complex, including FAK, p-FAK-Tyr^{397}, p-FAK-Tyr^{576}, p130Cas, p-p130Cas-Tyr^{410}, Crk, DOCK180, R-ras, RhoA, activated RhoA, Grb2 and vinculin. (B) Bar graphs herein are summarized data of (A) from 3 independent experiments. Each bar is the mean ± SD of n = 3 rats. *significantly different by ANOVA, $p < 0.05$; **$p < 0.01$; ns, not significantly different, nd, non-detectable.

p-p130Cas-Tyr^{410}, R-ras, RhoA, Grb2 decreased from 1 d onwards and became barely detectable by 15 d, whereas the protein level of p130Cas and Crk stayed high until 1 d and 4 d, respectively, and became barely detectable by 15 d (**Fig. 7A and B**). DOCK180 remained at a relatively steady level at the beginning of the treatment until it peaked at 8 h and then gradually decreased from 1 d onwards and became barely detectable by 15 d (**Fig. 7A and B**). While the level of FAK remained relatively unchanged from 0 h to 8 h but a significant reduction was detected at 1 d post treatment and became barely detectable by 15 d (**Fig. 7A and B**). The induced RhoA level was also accompanied by a significant increase in its intrinsic activity as assessed by the level of Rho A-GTP (**Fig. 7A and B**).

Immunohistochemical localization of β1-integrin, p130Cas, RhoA and vinculin in the seminiferous epithelium of rat testes during adjudin-induced anchoring junction disruption. Immunohistochemical localization of β1-integrin, p130Cas, RhoA and vinculin in the seminiferous epithelium after adjudin treatment was performed using cross-sections of treated rats at selected time points. Immunohistochemistry for DOCK180 was not included because the anti-DOCK180 antibody failed to yield satisfactory results based on preliminary experiments.

β1-integrin. In rat testes at time 0 (control, normal rat testes), β1-integrin was restricted almost exclusively to the apical ES in the epithelium, mostly abundantly in early stage VIII tubules (**Fig. 8A**). This pattern of localization of β1-integrin did not alter much by 1 hour (H) post-treatment since it was reported that rats treated with adjudin by gavage required ~1–3-hr before the drug could reach the

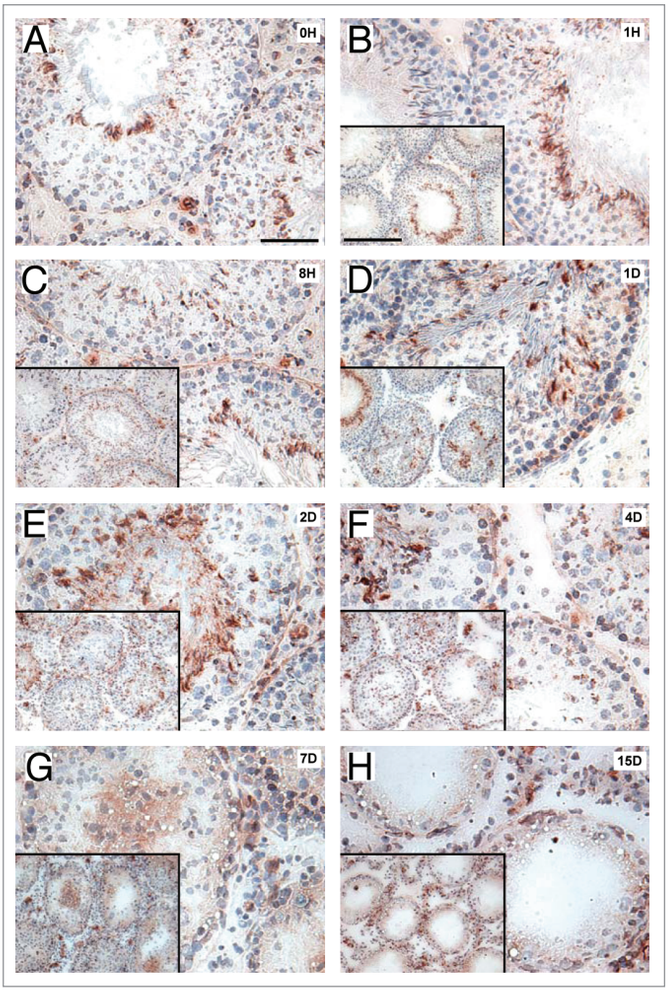

Figure 8. Changes in the localization and expression of β1-integrin in the seminiferous epithelium of adult rat testes during adjudin-induced anchoring junction restructuring and germ cell loss from the seminiferous epithelium. These micrographs are the results of a representative immunohistochemistry study of 3 independent experiments (with different sets of treated rats versus controls) which yielded similar data, illustrating the localization pattern of β1-integrin (reddish brown precipitates) in the seminiferous epithelium from normal rat testes at 0H (A), 1H (B), 8H (C), 1D (D), 2D (E), 4D (F), 7D (G) and 15D (H) after adjudin treatment (1 dose, 50 mg/kg b.w., by gavage). The boxed areas shown in (B–F) are cross-sections of the testes at lower magnification. Bar in (A) = 50 μm, which applies to (B–H); bar in boxed area in (B) = 250 μm, which applies to boxed areas in (C–H).

testes to exert its effects in the apical compartment of the seminiferous epithelium to perturb cell adhesion function,[29] initially at the apical ES to induce early spermatid loss from the epithelium (and by ~6-hr post treatment, about 50% of the tubules had elongating spermatids found in the tubule lumen illustrating apical ES disruption), to be followed by round spermatids (~3 d post treatment, with 50% of the tubules had round spermatids found in the tubule lumen) and spermatocytes (~6 d post treatment, with 50% of the tubules had spermatocytes found in the tubule lumen).[28] By 8 h, 1 d and 2 d, the expression of β1-integrin at the apical ES was considerably enhanced (**Fig. 8C–E** versus **A and B**) in tubules where elongating spermatids were induced to deplete from the epithelium even in a stage V (**Fig. 8D**) and VI (**Fig. 8E**) tubule. However, the expression and localization of β1-integrin in the epithelium was considerably diminished by 4, 7 and 15 days (**Fig. 8F–H**) when elongating spermatids (as well as round spermatids and many spermatocytes) were all depleted from the epithelium, illustrating the enhanced expression of β1-integrin in the epithelium at 8 h–2 d might have been used to 'retain' elongating spermatids in the epithelium by strengthening the apical ES adhesive function. These results of immunohistochemistry are also consistent with data obtained by immunoblot analysis shown in **Figure 7** that illustrate a transient increase in the steady-state protein level of β1-integrin.

p130Cas. For p130Cas, it was confined mostly to the apical ES in stage VIII tubules in control rat testes (**Fig. 9A**). However, during adjudin-induced anchoring junction disruption, p130Cas was found to be abundantly present at the apical ES in tubules that displayed signs of premature elongating spermatid release since elongating spermatids were found in the tubule lumen of apparently stage VII (**Fig. 9B**), V (**Fig. 9C**) and VI (**Fig. 9D**) tubules, and p130Cas was strongly associated with elongating spermatids in these tubules. Also, p130Cas was also associated with elongating spermatids that had already been depleted from the epithelium but found in the tubule lumen by day 4 (**Fig. 9E**). However, the expression of p130Cas was greatly diminished in the epithelium by 15 d when virtually all the elongating spermatids, spermatids and spermatocytes were depleted from the epithelium except spermatogonia (**Fig. 9F**).

RhoA. Similar to β1-integrin and p130Cas, RhoA was found at the apical ES, and most abundantly at stage VIII tubule prior to spermiation in normal testes at time 0 (**Fig. 10A**). However, during adjudin-induced anchoring junction restructuring and germ cell loss from the epithelium, RhoA was considerably expressed at the apical ES in stage VII tubules at 1H (**Fig. 10B**) and 8 H (**Fig. 10C**) post adjudin treatment, but also in stage V and VI tubules by 1 day after treatment (**Fig. 10D**). RhoA was also found to associate with departing elongating spermatids in tubule lumen by day 4 (**Fig. 10E**) but greatly diminished by day 15 when virtually all germ cells were depleted from the epithelium except spermatogonia and some early primary spermatocytes (**Fig. 10F**).

Vinculin. Similar to β1-integrin, p130Cas and Rho A, vinculin was found at the apical ES in normal rat testes at time 0 (**Fig. 11A**) and its expression was induced in departing elongating spermatids in the epithelium as well as those found in the tubule lumen (**Fig. 11B–D**) during adjudin-induced apical ES disruption and elongating spermatid loss from the epithelium. However, it is of interest to note that, unlike β1-integrin, p130Cas and RhoA, vinculin was also detected at the BTB, likely at the basal ES in normal testes (**Fig. 11A**). Interestingly, the expression of vinculin is considerably enhanced by day 4–15 in the basal compartment when virtually all the elongating spermatids were depleted from the epithelium (**Fig. 11E and F**). This surge in vinculin expression perhaps is being used to "reinforce" the adhesion function at the BTB to maintain the integrity of the immunological barrier since it is noted that amidst the disruption of anchoring junction function in the seminiferous epithelium at the Sertoli-germ cell

interface, the BTB remained intact by 2-day post adjudin treatment at 50 mg/kg b.w. even though the tubules were devoid of virtually all germ cells in the epithelium.[74,77,78]

Discussion

The β1-integrin-FAK protein complex at the apical ES. It is known that FAK is a common mediator for transducing signals originated from integrins at the focal adhesion complex (also known as focal contact) in most epithelia to facilitate cell migration, such as fibroblasts and macrophages.[30-33] Thus, FAK is crucial to regulate integrin originated signaling function at the actin-based cell-matrix anchoring junction known as focal adhesion complex or focal contact. In fact, β1-integrin is known to interact with FAK near its N-terminus within the FERM (band 4.1, ezrin, radixin, moesin homology) domain.[31,33-35] Besides c-Src, FAK is one of the most important non-receptor tyrosine protein kinases that mediates a number of cellular functions including gene expression, cell survival, apoptosis, cell growth and movement,[34,35] including tumorigenesis.[36,37] This is possible because, besides the catalytic kinase domain (that confers FAK its intrinsic protein kinase activity) and the FERM domain, FAK also possesses a FAT (focal adhesion targeting) domain near its C-terminus as well as several proline-rich regions I, II and III (PRI–PRIII), which serve as protein-protein interaction sites to recruit additional kinases and/or adaptors to FAK to execute diversified cellular events to regulate cell homeostasis in response to changes in environment or external stimuli.[15,30,31,34] However, we were surprised with our initial observation regarding the presence of p-FAK-Tyr^{397} and p-FAK-Tyr^{576} at the apical ES, which is a testis-specific atypical AJ type instead of focal contact, and FAK was also shown to be an integrated component of the integrin-based cell adhesion complex at the apical ES based on studies using Co-IP.[16] Since conventional wisdom would expect that FAK to be limited to the focal adhesion complex (or focal contact) at the cell-extracellular matrix interface as in other epithelia[30] (note: focal contacts are not found in the mammalian testis), thus, the earlier observation that FAK is a component of the apical ES,[16] an actin-based cell-cell anchoring junction type, made it difficult to appreciate the significance of these findings. Nonetheless, subsequent studies from another laboratory have confirmed this earlier observation that p-FAK is indeed tightly associated with the apical ES in rat testes[18] and it is critical to be involved in the process of sperm release during spermiation.[5] Additionally, we have also demonstrated in recent studies that FAK is also an integrated component of the occludin-ZO-1 complex, regulating the Sertoli cell tight junction-permeability barrier at the Sertoli-Sertoli cell interface.[38,39] Herein, we further expand our earlier observations to identify other components of this β1-integrin-FAK cell adhesion complex at the apical ES, illustrating this complex may be used to induce rapid "adhesion" and "de-adhesion" at the β1-integrin-based apical ES during spermiogenesis to facilitate changes in spermatid cell shape and its relative position in the seminiferous epithelium via the action of FAK-induced phosphorylation and the RhoA-mediated cytoskeletal dynamics. In this context, it is of interest to note that c-Src was also shown

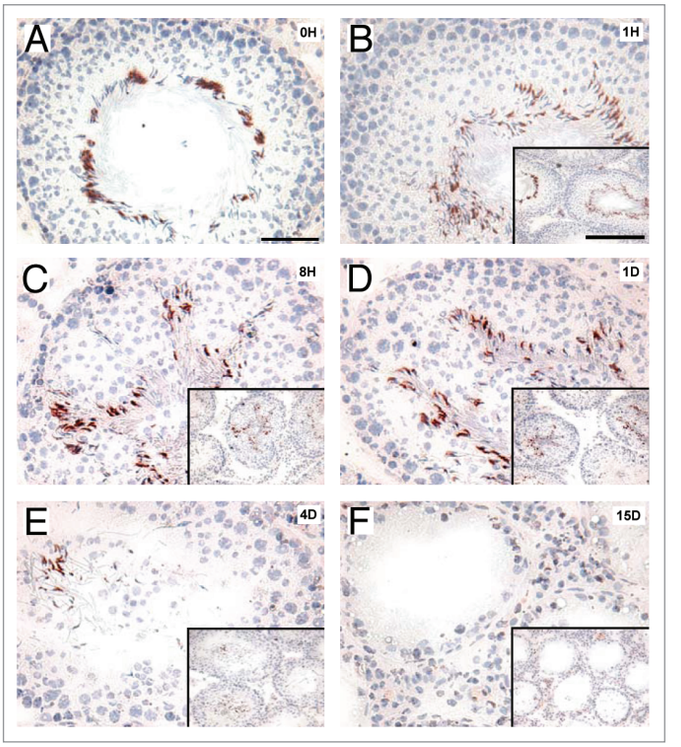

Figure 9. Changes in the localization and expression of p130Cas in the seminiferous epithelium of adult rat testes during adjudin-induced anchoring junction restructuring and germ cell loss from the seminiferous epithelium. This experiment was performed similar to the one shown in **Figure 8** except that the localization and expression of p130Cas was examined. These micrographs are representative data from a single experiment which was repeated three times using different sets of treated rats versus control rats at time 0 and yielded similar results. The localization pattern of p130Cas (reddish brown precipitates) in the seminiferous epithelium from normal rat testes at 0H (A), 1H (B), 8H (C), 1D (D), 4D (E) and 15D (F) after adjudin treatment (1 dose, 50 mg/kg b.w., by gavage). The boxed areas shown in (B–F) are cross-sections of the testes at lower magnification. Bar in (A) = 50 μm, which applies to (B–F); bar in boxed area in (B) = 250 μm, which applies to boxed areas in (C–F).

to structurally interact with β1-integrin and FAK at the apical ES[16] and p-c-Src-Tyr^{416} was found to be intensely localized to the apical ES at stage VIII of the epithelial cycle,[40] and other studies have shown that the dual c-Src/FAK kinase complex is a crucial regulator of many epithelial cell function including the testis.[41,42] Thus, FAK and c-Src at the apical ES may confer the necessary phosphorylation status to β1-integrin- and perhaps cadherin- and nectin-based cell adhesion protein complexes during spermiogenesis. This possibility must be carefully evaluated in future studies.

β1-Integrin-p-FAK-p130Cas-DOCK180-RhoA-vinculin is a novel regulatory complex of cell adhesion at the apical ES in the seminiferous epithelium. Besides p-FAK, we have now demonstrated that p130Cas, DOCK180, RhoA and vinculin are also integrated components of the β1-integrin-based protein complex at the apical ES. p130Cas [p130 Crk-associated substrate, an adaptor protein encoded by Crk-associated (Crkas) gene, a substrate for src family kinases, also known as BCAR1 (breast cancer anti-estrogen resistance 1), is a member of the CAS

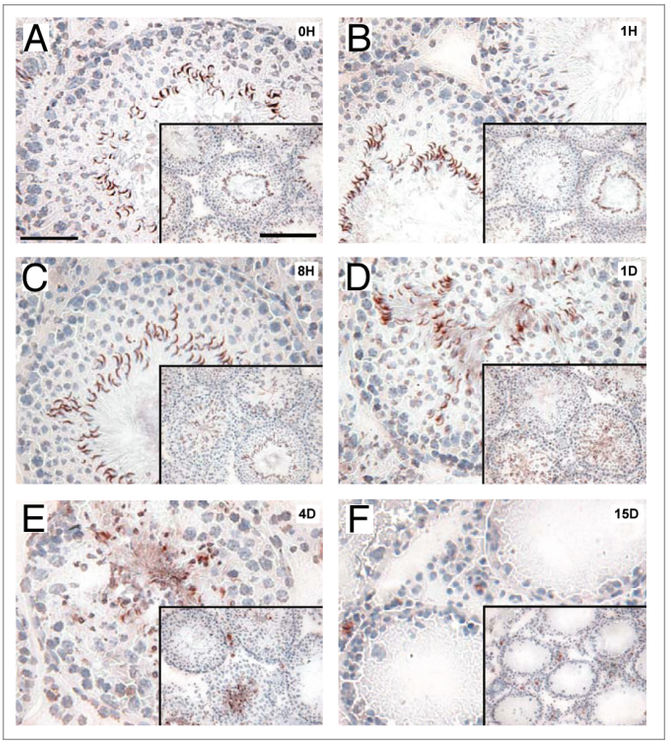

Figure 10. Changes in the localization and expression of RhoA in the seminiferous epithelium of adult rat testes during adjudin-induced anchoring junction restructuring and germ cell loss from the seminiferous epithelium. These micrographs are representative data from a single experiment which was repeated three times using different sets of treated rats versus control rats at time 0 and yielded similar results. The localization pattern of RhoA (reddish brown precipitates) in the seminiferous epithelium from normal rat testes at 0H (A), 1H (B), 8H (C), 1D (D), 4D (E) and 15D (F) after adjudin treatment (1 dose, 50 mg/kg b.w., by gavage). The boxed areas shown in (A–F) are cross-sections of the testes at lower magnification. Bar in (A) = 50 μm, which applies to (B–F); bar in boxed area in (A) = 250 μm, which applies to boxed areas in (B–F).

(Crk-associated substrate) family; note: Crk is an oncogene originally identified in chicken sarcoma, encoding an activator of protein tyrosine kinase] is an adaptor (or scaffold) protein known to regulate cell cycle, apoptosis, cell movement and differentiation in multiple epithelia, mostly at the focal contacts.[43,44] DOCK180 (Dedicator of cytokinesis 180, also known as DOCK1, a member of the DOCK family of guanine nucleotide exchange factors, GEFs) was initially identified as a binding protein for the SH3 domain of the proto-oncogene c-Crk which is mediated via its C-terminal PXXP region.[45] DOCK180 is genetically conserved from *C. elegans* and Drosophila to mammals, and subsequent studies have shown that DOCK180 and DOCK180-related proteins are crucial regulators of GTPases (e.g., Rac and Cdc42 of the Rho GTPase family) since it contains a DOCK homology region-2 domain that catalyzes guanine nucleotide exchange on Rho GTPases, serving as nucleotide exchange factors (GEFs) for Rho GTPase activation, regulating cytoskeletal dynamics, cell polarity and movement and differentiation.[46,47] Vinculin is an peripheral adaptor protein having binding sites for more than 15 proteins, involving in cell signaling, cell adhesion, cytoskeletal dynamics, but also mostly at focal contacts.[48,49] However, we have shown herein that vinculin is also a component of the basal ES at the BTB besides it intense stage-specific expression and localization at the apical ES. Collectively, p130Cas, DOCK180, RhoA and vinculin [and their associated partners, such as Crk, R-ras (a GTPase involved in Rho signaling, regulating actin dynamics[50,51]) and Grb2 (growth factor receptor-bound protein 2, also an adaptor involved in the p130Cas/DOCK180 signaling[52,53])] are putative components of the β1-integrin-based adhesion protein complex at the apical ES in the seminiferous epithelium. In this context, it is noted that the trend of activation of Crk (proto-oncogene c-Crk, an adaptor coded by CRK gene[54]) is analogous to DOCK180 and/or p130Cas during apical ES assembly in Sertoli-germ cell cocultures or adjudin-induced anchoring junction disruption since Crk interacts with p130Cas and DOCK180 via its SH2 and SH3 domain, respectively.[44] In short, the findings reported herein suggest that the binding of β1-integrin to p-FAK create a unique protein complex, possibly following the activation of FAK at Tyr-[397] and Tyr-[576], which in turn, recruit p130Cas, DOCK180 and vinculin, involving also Crk, R-ras and Grb2, to activate RhoA, modulating the actin-based cytoskeletal network at the apical ES to facilitate the cellular events of spermiogenesis. Since the most prominent and unique ultrastructural feature of the apical ES is the highly organized actin filament bundles that are sandwiched in between the cisternae of endoplasmic reticulum and the apposing plasma membranes of the Sertoli cell and the elongating spermatid,[4,55] perhaps being used to confer the adhesive function at the apical ES. It is likely that this β1-integrin-p-FAK-p130Cas-DOCK180-RhoA-vinculin complex exerts its effect to modulate the "plasticity" and "fluidity" of the actin filament bundles, perhaps working in concert with other actin regulatory proteins (e.g., Eps8 and Arp2/3 complex),[56] thereby facilitating the "adhesion" and "de-adhesion" of the apical ES in the epithelium to coordinate the timely transit of developing spermatids across the seminiferous epithelium during spermiogenesis.

The downstream signaling pathway of this novel β1-integrin-pFAK-p130Cas-DOCK180-RhoA-vinculin complex. The downstream activation pathway of this novel β1-integrin-FAK-based protein complex is presently unknown. However, it appears to involve ERK since p-ERK was shown to be activated in studies[57,58] using the models of Sertoli-germ cell cocultures in vitro and of anchoring junction restructuring in vivo model induced by adjudin as reported herein, and since the primary cellular target of adjudin is the apical ES,[59,60] this possibility is highly plausible and should be vigorously investigated. The finding regarding the activation of ERK during apical ES disruption, such as induced by adjudin, is also consistent with recent studies using an anti-spermatogenic indenopyridine, I-CDB-4022, to induce anchoring junction restructuring that led to spermatid loss from the seminiferous epithelium in adult rats.[61] It is also consistent with an earlier report using TE implants to suppress intratesticular androgen to induce spermatid release from the epithelium as a result of apical ES disruption, which also led to an activation of p-ERK.[40,58,62] The findings reported herein are significant since they illustrate for the first time that the apical ES,

which is the only anchoring device at the Sertoli cell-elongating spermatid interface during spermiogenesis in step 8–19 spermatids, is using some of the most efficient regulatory/signaling molecules and the MAPK signaling pathway commonly found at the focal contact site to regulate cell movement,[30,31] perhaps being used to facilitate the transit of elongating spermatids across the seminiferous epithelium during spermiogenesis. Since there are inhibitors or modulators against many of these non-receptor protein kinases, such as p-FAK, DOCK180 and RhoA, it seems that spermiogenesis, spermatid adhesion and/or spermatid polarity/orientation can be manipulated by targeting some of these regulatory components in the integrin-based adhesion protein complex.

Summary and concluding remarks. In summary, we have demonstrated that the p130Cas-DOCK180-RhoA-vinculin complex is a functional component of the β1-integrin-p-FAK (or possibly the β1-interin-p-FAK/p-Src) complex, which regulates the adhesion function of the β1-integrin-based adhesion protein complex at the apical ES, facilitating spermatid transit across the seminiferous epithelium during spermiogenesis. Functional studies can now be designed to delineate the precise physiological role of each component protein in this complex to regulate apical ES dynamics.

Materials and Methods

Animals. Sprague-Dawley rats were purchased from Charles River Laboratories (Kingston, NY). The use of rats for the studies reported herein was approved by the Rockefeller University Animal Care and Use Committee with Protocol Numbers 00111, 03017, 03040 and 06018. Adult rats (~300 gm b.w.) were housed with two rats per cage, and male pups were housed with 10 pups per cage with a foster mother. All rats had free access to standard chow and water, and housed in a temperature controlled environment of 22°C with a constant light-dark cycles (12 hr:12 hr) at the Rockefeller University Comparative Bioscience Center.

Seminiferous tubule cultures and lysate preparation. Seminiferous tubules with negligible Leydig and peritubular myoid cell contamination were isolated from adult rat testes as earlier described.[63,64] Lysates were prepared using an IP buffer [0.125 M Tris, pH 7.4 at 22°C containing 1% NP-40 (v/v), 2 mM EGTA, 2 mM N-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate and 0.1 μM sodium okadate]. Samples were centrifuged at 15,000 g for 20 min to remove cellular debris, and clear supernatants were obtained and stored at -80°C until used.

Primary sertoli cell cultures. Sertoli cells isolated from 20-day-old rat testes were plated at high cell density (0.5 x 10^6 cells/cm^2) on Matrigel-coated 12-well dishes in serum-free F12/DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with growth factors as described.[65] It is noted Sertoli cells isolated from 20-day-old rats were differentiated and ceased to divide, and its functional characteristics were shown to be similar to Sertoli cells isolated from adult rat testes using BSA gradientas earlier reported.[66,67] When cultured in vitro on Matrigel-coated bicameral units, a functional TJ-permeability barrier was detected within 24–48 hr when assessed by transepithelial electrical resistance (TER) measurement across the Sertoli cell epithelium and ultrastructures of the BTB that mimicked the BTB in vivo, such as TJ, basal ES and desmosome were also visible by electron microscopy at this cell density.[65,68] About 48 hr after plating, cells were hypotonically treated with 20 mM Tris (pH 7.4, 2.5 min) to lyse residual germ cells.[69] The cell purity was greater than 98% with negligible contamination of germ cells, peritubular myoid cells, and/or Leydig cells using corresponding specific markers.[70] After 4 days, cells were either lysed in a SDS Lysis Buffer [0.125 M Tris, pH 6.8 at 22°C containing 1% SDS (w/v), 2 mM EGTA, 2 mM N-ethylmaleimide, 2 mM PMSF, 1.6% 2-mercaptoethanol (v/v), 1 mM sodium orthovanadate and 0.1 μM sodium okadate] for immunoblot analysis or used for Sertoli-germ cell cocultures.

Sertoli-germ cell cocultures. Total germ cells isolated from adult rat testes including spermatogonia, spermatocytes, round

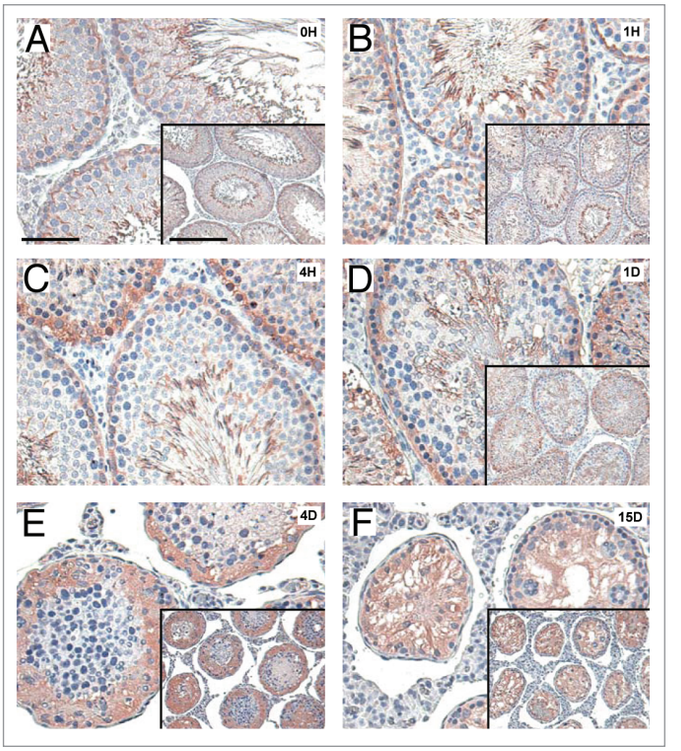

Figure 11. Changes in the localization and expression of vinculin in the seminiferous epithelium of adult rat testes during adjudin-induced anchoring junction restructuring and germ cell loss from the seminiferous epithelium. These micrographs are representative data from a single experiment which was repeated three times using different sets of treated rats versus control rats at time 0 and yielded similar results. In this experiment, paraffin sections of testes were used for immunohistochemistry for the localization of vinculin in the seminiferous epithelium (see Materials and Methods). The localization pattern of vinculin (reddish brown precipitates) in the seminiferous epithelium from normal rat testes at 0H (A), 1H (B), 4H (C), 1D (D), 4D (E) and 15D (F) after adjudin treatment (1 dose, 50 mg/kg b.w., by gavage). The boxed areas shown in (A, B and D–F) are cross-sections of the testes at lower magnification. Bar in (A) = 50 μm, which applies to (B–F); bar in boxed area in (A) = 250 μm, which applies to boxed areas in (B and D–F).

and elongating/elongated spermatids at a relatively ratio similar to that in vivo when assessed by flow cytometry[71] were added (at time 0) onto the Sertoli cell epithelium, which had been cultured alone for 3 days to establish a functional Sertoli cell TJ-permeability barrier, and cocultured at a Sertoli:germ cell ratio of 1:1 (on day 4, but treated as time 0 in the coculture experiments) to allow the assembly of apical ES as described elsewhere.[65] Functional apical ES was also detected in these Sertoli-germ cell cocultures by electron microscopy as detailed elsewhere in reference 65. Cocultures were terminated at specific time points by the SDS Lysis Buffer for immunoblot analysis or by a Mg^{2+} Lysis Buffer [125 mM HEPES, pH 7.5 at 22°C, containing 750 mM NaCl, 5% IGEPAL CA-630 (octylphenoxypolyethoxyethanol, a nonionic and non-denaturing detergent, v/v), 50 mM $MgCl_2$, 5 mM EDTA, 10% glycerol (v/v), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 mM sodium fluoride and 1 mM sodium orthovanadate] for intrinsic kinase assay. Protein estimation was performed by Coomassie blue dye-binding assay using BSA as a standard as earlier described.[72]

Treatment of rats with adjudin. Adjudin [1-(2,4-dichlorobenzyl)-*1H*-indazole-3-carbohydrazide, formerly known as AF-2364] is known to perturb Sertoli-germ cell adhesion function leading to premature germ cells loss from the epithelium, causing reversible infertility in rats.[29,60] Apical ES is one of the primary targets of adjudin[73] since the loss of elongating spermatids occurred before round spermatids and spermatocytes,[28] but the spermatogonia (and/or spermatogonial stem cells were largely unaffected).[74] Aowever the BTB integrity remains unaffected in these rats by 2-wk following adjudin treatment.[75-78] Adult rats were fed with a single dose of adjudin [with adjudin suspended in 0.25% methycellulose (w/v, in MilliQ water) as a 20 mg/ml stock] at 50 mg/kg b.w. at time 0.[29] Testes were removed from rats (n = 3–6 rats for each time point) at specified time points and lysates were obtained by homogenization either in the SDS Lysis Buffer (for immunoblot analysis) or Mg^{2+} Lysis Buffer (for Rho A activation assay). For immunohistochemistry, testes were immediately fixed in 4% paraformaldehyde prior to paraffin embedding and sectioning.

Co-immunoprecipitation (Co-IP) and immunoblot analysis. Co-IP was performed as earlier described.[65] Primary antibodies used Co-IP are listed in **Table 1**. Lysates of seminiferous tubules without incubation with any antibodies or incubated with normal serum were used and served as positive controls in Co-IP. Immunoblot analysis was performed as described.[65] The source and working dilutions of primary and secondary antibodies are listed in **Table 1**. Immunoblot analysis was performed as described,[65] using antibodies obtained from different vendors and listed in **Table 1**.

Rho A activation assay. To assess the intrinsic Rho A activity during anchoring junction assembly in Sertoli-germ cell cocultures and during adjudin-mediated Sertoli-germ cell anchoring junction disruption in the testis, Rho activation assay kit (Upstate Biotechnology, Hamburg, Germany, Catalog # 17-294) and an anti-Rho A antibody (see **Table 1**) were used according to the manufacturer's protocol. In brief, lysates of Sertoli-germ cell cocultures and adjudin treated testes prepared as described above were incubated with the GST-Rho-binding domain of rhotekin (RBD) beads for 45 minutes at 4°C to isolate the activated RhoA (i.e., RhoA-ATP), washed three times with Mg^{2+} lysis buffer, and then eluted with SDS sample buffer. Activated Rho A protein (i.e., the RhoA-GTP bound form) was subsequently detected with an anti-Rho A antibody by immunoblot analysis. Positive and negative controls were also included in the Rho A activation assay as described in the manufacturer's protocol.

Immunohistochemistry. Immunohistochemistry was performed as described,[65] using Histostain SP kits [Broad Spectrum, 3-amino-9-ethylcarbazole (AEC), Invitrogen/Zymed]. For the staining of β1-integrin, p130Cas and RhoA, frozen sections were used. Testes removed from rats were immediately frozen in liquid nitrogen, embedded in O.C.T. (optimal cutting temperature) compound (Miles Scientific, Elkhart IN), and stored at -80°C until used. Frozen sections (6 μm thick) were cut in a cryostat (at -20°C), mounted on poly-L-lysine (Sigma, Mr >150 kDa) coated slides, air-dried at room temperature, and then fixed in modified Bouin's fixative (4% formaldehyde in picric acid) for 5 min, and washed thoroughly with PBS (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 at 22°C). For the staining of vinculin, paraffin sections were used. Testes were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin and sectioned. Following de-paraffinization, antigen recovery was performed by heating sections at temperature around boiling in 10 mM sodium citrate buffer, pH 6.0 for 10 min and then cooled for 20 min at room temperature. Streptavidin-biotin-peroxidase immunostaining was performed. Fixed sections were treated with 3% hydrogen peroxide in methanol (v/v), and then subsequently incubated with serum blocking solution (Zymed), to be followed by corresponding primary antibodies (see **Table 1**), biotinylated goat anti-mouse IgG or goat anti-rabbit IgG-peroxidase, and then streptavidin-peroxidase, and then stained with AEC mixture, counterstained in hematoxylin and mounted. Sections were photographed using an Olympus BX-40 microscope (Olympus Corp., Melville, NY). Images were acquired using an Olympus QColor 3 cooled digital camera and the QCapture (V1.2.0) Software Package (Quantitative Imaging Corp.,), and analyzed by Adobe Photoshop (Version 7.0). Negative control was performed by substituting the primary antibody with preimmune IgG of either rabbit or mouse origin.

Statistical analysis. All experiments were repeated at least three times using different sets of cultures, cocultures and animals for statistical analysis. To compare selected pairs of experimental groups, statistical analyses were performed by two-way analysis of variance (ANOVA) using the repeated measures model to be followed by Dunnett's test to compare changes between treatment groups and their corresponding controls using the GB-STAT statistical analysis software package (Version 7, Dynamic Microsystems, Silver Spring, MD). In short, changes in selected parameters (e.g., steady-state level of a target protein) in treatment groups versus the corresponding controls (first variable) and also as a function of time (second variable) were taken into consideration in statistical analysis.

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